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Xenotransplantation of uterine leiomyoma in Wistar rats: a pilot study

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ABSTRACT

Objective: To evaluate whether xenografts derived from hysterectomized patients would implant successfully and lead to uterine leiomyoma in Wistar rats.
Study design: This experimental study examined six female Wistar rats implanted with uterine leiomyoma obtained from patients who underwent hysterectomies at the gynecological surgery service of the HUUFMA. The rats were divided into two groups. Group I consisted of three rats in which the uterine leiomyoma had been implanted in the parietal peritoneum, and group II consisted of three rats in which the uterine leiomyoma was implanted in the subcutaneous tissue. The immunosuppressant mycophenolate mofetil (MMF) was administered orally by gavage (at a dose of 40 mg/kg of body weight) to prevent transplant rejection starting 15 days before the transplant and continuing throughout the entire experiment. After four weeks, necrosis and neovascularization were evaluated histologically in both groups and were classified as either absent or present. Lymphocytic inflammatory infiltration was also examined and classified as mild, moderate or intense (by hematoxylin and eosin staining), and fibrosis was classified as grade I–III (by Masson's trichrome staining).
Results: Necrosis was absent from all three rats in group I and was observed in only one rat from group II. Neovascularization was present in two rats from group I and in only one rat from group II. The lymphocytic inflammatory infiltrate was mild in two rats and moderate in one rat from group I, and it was moderate in two rats and intense in one rat from group II. Two rats from group 1 exhibited grade III fibrosis, and one rat presented grade I fibrosis. In group II, two rats presented grade I fibrosis and one rat had grade II fibrosis. When necrosis and neovascularization were evaluated as variables, group I demonstrated greater evidence of successful implantation when compared to group II, indicating that the peritoneal implantation technique produces better results than the subcutaneous approach ($p = 0.039$).
Conclusion: This study demonstrates that the xenotransplantation of uterine leiomyoma into the parietal peritoneum is more effective than the xenotransplantation of uterine leiomyoma into the subcutaneous tissue, and it describes a promising new model for the study of leiomyoma *in vivo*.

Introduction

Leiomyomas are the most common type of solid pelvic tumor, and they affect approximately 20–40% of reproductive-age women [1,2]. Despite this subject being thoroughly studied, the optimal clinical treatments for this disease remain unknown. Thus, the development of appropriate experimental models to study potential new treatment options may be important for improving treatment options [3–6].

Experimental models of uterine leiomyoma in which patient-derived xenografts are transplanted into female rats have been proposed. However, the studies using these models, which range from simple genetic manipulations to highly complex methods, have been characterized by problems with reproducibility [7–12].

Preventing transplant rejection is a key step in successful transplantations that can be achieved with drug therapy, among

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other measures. Mycophenolate mofetil (MMF), an inhibitor of nucleotide synthesis, is among the drugs used to prevent acute transplant rejection in transplantation surgeries. RS-61443 is a semi-synthetic morpholinoethyl ester pro-drug that is hydrolyzed to mycophenolic acid. This drug acts by blocking purine synthesis [13], thus reducing nucleotide synthesis. Xenotransplantation in hamsters using immunosuppressive therapy with MMF has been shown to be satisfactory in studies with cardiac tissue [14] and porcine islet-like cell clusters [15,16].

Despite all current knowledge that has been obtained regarding the pathophysiology of uterine leiomyomas, there are no conservative treatments or drugs that have been proven to be effective for this condition. Animal models are required to study potential new therapeutic options. This study sought to develop a reliable *in vivo* model of human uterine leiomyoma using Wistar rats with immunosuppressant mycophenolate mofetil to enable and contribute to future studies investigating new treatments.

Materials and methods

This pilot experiment used Wistar rats (*Rattus norvegicus albinus*) obtained from the animal facility of the Federal University of Maranhão (Universidade Federal do Maranhão – UFMA) implanted with uterine leiomyomas removed from women with symptoms of leiomyoma who were subjected to hysterectomy at the Mother and Child Unit of the University Hospital (Hospital Universitário – Unidade Materno Infantil – HUUMI). All surgical procedures were performed in the surgical center or the Experimental Surgery Laboratory of Maranhão (Laboratório de Cirurgia Experimental do Maranhão – LabCEMA) at the HUUMI between May and July 2014.

This study was performed in accordance with Brazilian law for the use of experimental animals (Arouca Law No. 11,794/2008) and the guidelines from the Brazilian College of Animal Experimentation (Colégio Brasileiro de Experimentação Animal – COBEA), an institution affiliated with the International Council for Laboratory Animal Science. It was approved by the Animal Ethics Committee (Comitê de Ética de Uso de Animais – CEUA) of the Federal University of Maranhão and the Research Ethics Committee (Comitê de Ética e Pesquisa – CEP) of the University Hospital – UFMA in agreement with the requirements of Law No. 11,794 from October 8, 2008.

Six 60-day-old adult virgin female Wistar rats weighing between 180 and 250 g that presented four or more five-day estrous cycles were used in the study. The animals were placed in polypropylene cages (three rats per cage) with a stainless steel grill lid that measured 46 cm × 31 cm × 16 cm. Sawdust was used for the bedding material, which was changed every 48 h. The animals were kept under controlled environmental conditions with food (rat chow, Purina®, São Paulo, Brazil) and water available *ad libitum*. Noise levels were controlled, the temperature was maintained at 22 °C ± 2 °C, the relative humidity was kept between 40 and 60% and a 12:12 light–dark cycle was utilized starting seven days prior to the start of experiments to allow for acclimation.

The immunosuppressant mycophenolate mofetil (Cellcept® 500 mg) was administered by oral gavage (at a dose of 40 mg/kg body weight) starting 15 days before the xenotransplantation procedure and continuing throughout the entire experiment to prevent transplant rejection [15,17].

Uterine tumors were obtained from patients undergoing a hysterectomy at the HUUMI due to symptoms of multiple uterine leiomyomas. The patients were recruited from the general gynecology outpatient clinic based on indications for surgical treatment that were observed during the standard preoperative routine provided by HUUMI. Patients diagnosed with infectious diseases, such as HIV, syphilis, hepatitis B and hepatitis C were excluded from the study. After the patients were selected and informed about the study, they signed an informed consent form accepting their inclusion in the study.

Abdominal hysterectomy was performed under anesthesia. There was no discrimination regarding the location (submucosal, intramural or subserosal) of the fibroids. Subsequently, tumors measuring 3–5 mm in diameter were selected, avoiding the need of unnecessary trauma to the myoma so that it could be transplanted whole. The remaining tumors and excess material was sent along with the uterus for histopathological assessment using routine pathology protocols. After the removal and confirmation of the uterine leiomyoma, the sample identified with the patient's name was placed in normal saline solution and transported to the Experimental Surgery Laboratory of Maranhão (LabCEMA) for the xenotransplantation. At this time, the recipient rat was also identified. The interval between the end of the hysterectomy and the beginning of the xenotransplantation procedure was approximately 15–30 min.

All rats were subjected to a *12-h fasting* period and weighed using an electronic scale with the values (in grams) recorded in the protocol. After weighing, the rats were anesthetized with a mixture of 2% xylazine and 5% ketamine at a ratio of 1:1 (0.2 ml for each 100 g body weight). The anesthetics were administered *intramuscularly into* the posterior border of the left thigh.

In group I, which consisted of three animals, the xenograft was surgically transplanted into the peritoneal cavity via the abdomen. This surgical technique involved a 3-cm midline incision that was made in the caudal third of the abdomen and cutting the skin, muscular aponeurotic plane and peritoneum, followed by the identification of the organs of the abdominal cavity. In this group, a piece of uterine leiomyoma was transplanted into the right side of the peritoneal cavity in the middle third of the incision (0.5 cm laterally) and was attached with a single stitch using 5–0 nylon sutures (Fig. 1A).

Group II also consisted of three animals, but the xenografts were transplanted subcutaneously for this group (Fig. 1B). For these transplant surgeries, a small 3-cm incision was made in the rat's skin in the caudal third of the right flank of the rat. Once the incision was made, the skin layer (upwards of 1.5 cm) was separated from the aponeurosis. Then, a piece of uterine leiomyoma was transplanted into the subcutaneous tissue of the rat.

After xenotransplantation, the animals were monitored daily for four weeks and maintained under constant environmental conditions. They were provided rat chow, the immunosuppressive therapy described above and estrogen supplementation with estradiol valerate (Prymogina 1 mg®). For the estrogen supplementation, one pill was macerated and diluted in 250 ml of water, which was available *ad libitum* and changed every 24 h.

After the procedure, the rats were euthanized via sodium thiopental administration (Tiopental®) and evaluated. Euthanasia was performed according to resolution No. 714 from June 20, 2002 of the Brazilian Federal Council of Veterinary Medicine. Death was characterized by respiratory arrest and the complete absence of reflexes [18].

After the confirmation of death, all animals were subjected to surgical approaches using the same initial incision, and the xenograft was assessed in both groups.

The tissues removed were rinsed with 0.9% sodium chloride and kept in a labeled container with 10% buffered formalin. Tissues were sent to the *Anatomic* Pathology Division of the University Hospital (UFMA) and embedding in a paraffin block, staining with Hematoxylin and Eosin (H&E) and *Masson's trichrome* staining. Finally, all tissues were subjected to histological evaluations using light microscopy by a pathologist.

Subsequently, slides from each group were examined by the pathology service and evaluated according to the following histological criteria: cell necrosis, neovascularization and lymphocytic

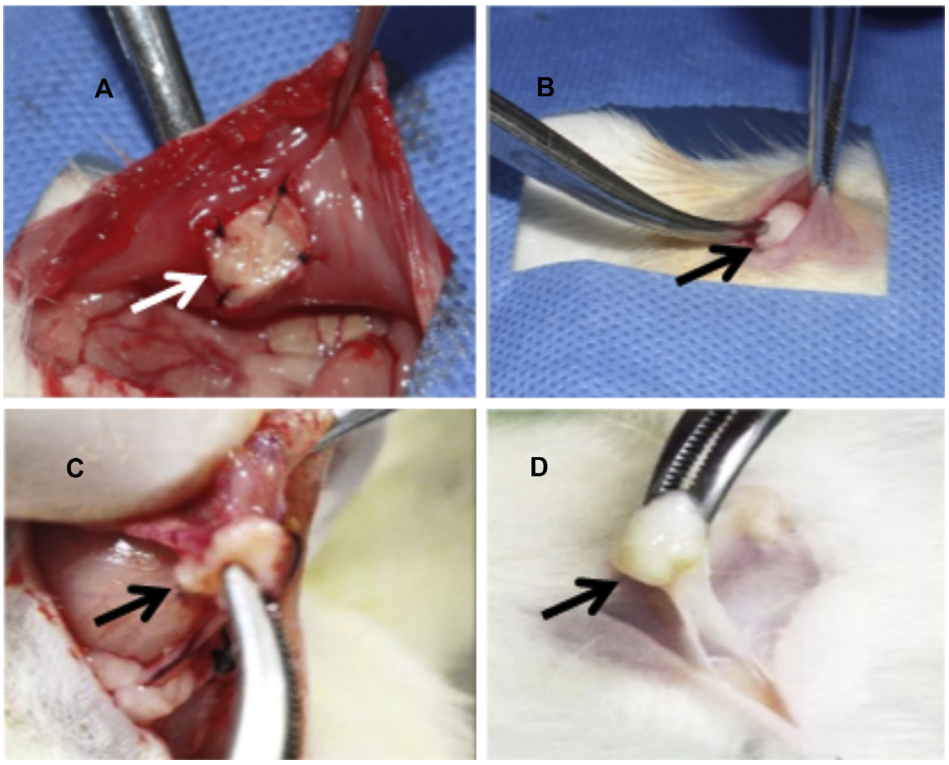

Fig. 1. (A) (group I) Implantation human uterine leiomyoma tissue into the peritoneal cavity via the abdomen (arrow); (B) (group II) the xenografts were transplanted subcutaneous (arrow). (C) Macroscopic aspect of a human uterine leiomyoma xenograft (arrow) in the parietal peritoneum (group I); (D) Macroscopic aspect of a human uterine leiomyoma xenograft (arrow) in subcutaneous tissue (group II). Both C and D images were taken four weeks after the transplant of human uterine leiomyoma in female Wistar rats that were immunosuppressed with mycophenolate mofetil (40 mg/kg) and supplemented with estrogen (estradiol valerate) during the entire experiment.

inflammatory infiltrate (by H&E staining) and the presence of fibrosis (by *Masson's trichrome* staining).

The presence or absence of necrosis was evaluated, and necrosis was only considered to be absent if it was observed in 0% of the cells on a given slide [19,20].

The analyses of neovascularization were assessed by the presence or absence of small tortuous vessels in the fibrin-leukocyte buffer [21].

Collagen and smooth muscle tissue (uterine fibroid) content was assessed using *Masson's trichrome* staining, and the presence of fibrosis was classified on a scale from I to III according to the staining intensity. The following criteria were used to measure fibrosis: grade I less than 33%, grade II between 34 and 66% and grade III between 67 and 100% [22].

The presence of lymphocytic inflammatory infiltration was classified as mild (<30%), moderate (31–60%) or intense (61–100%).

If the histopathological analysis revealed that the uterine leiomyoma was not benign, the donor patient would be informed about the test results to exclude the risk of malignancy.

Data were expressed as frequencies (absolute and relative) for the categorical variables shown in the tables and figures. Fisher's exact test was applied to correlate the categorical variables with the experimental group. Stata® software (version 12) was used for the analysis. Regarding the statistical interpretations of the results, the significance threshold *was set* at an alpha *value* of less than 0.05 for all tests.

Results

At the end of the experiment, all six rats were euthanized, three from group I and three from group II. During the experimental period (four weeks), no complications with the oral gavage, anesthesia or surgery were observed, nor were any postoperative complications, such as infections. The uterine fibroids that were xenotransplanted into the rats maintained the macroscopic characteristics of fibroid tissue in both groups (Fig. 1C and D).

Based on the histological evaluations by H&E staining, necrosis was absent in all three rats in group I, but it was absent in only one rat in Group II. Neovascularization was observed in two rats in group I but only one rat in group II. The degree of lymphocytic inflammatory infiltration was mild in two rats and moderate in one rat in group I, while it was moderate in two rats and intense in one rat in group II (Table 1).

Table 1
Histological variables of human uterine leiomyoma xenografts four weeks after being transplanted into the parietal peritoneum (Group I) and subcutaneous tissue (Group II) of female Wistar rats immunosuppressed with mycophenolate mofetil (40 mg/kg) and supplemented with estrogen (estradiol valerate) throughout the entire experiment.

Variable	Group I	Group II	*p*-value
Lymphocytic inflammatory infiltrate			
Mild	2 (66.7)	0 (0.0)	0.400
Moderate	1 (33.3)	2 (66.7)	
Intense	0 (0.0)	1 (33.3)	
Neovascularization			
Present	2 (66.7)	1 (33.3)	1.000
Absent	1 (33.3)	2 (66.7)	
Fibrosis grade			
Grade 1	0 (0.0)	2 (66.7)	0.600
Grade 2	1 (33.3)	1 (33.3)	
Grade 3	2 (66.7)	0 (0.0)	
Necrosis			
Present	0 (0.0)	2 (66.7)	0.400
Absent	3 (100.0)	1 (33.3)	
Total	3 (50.0)	3 (50.0)	

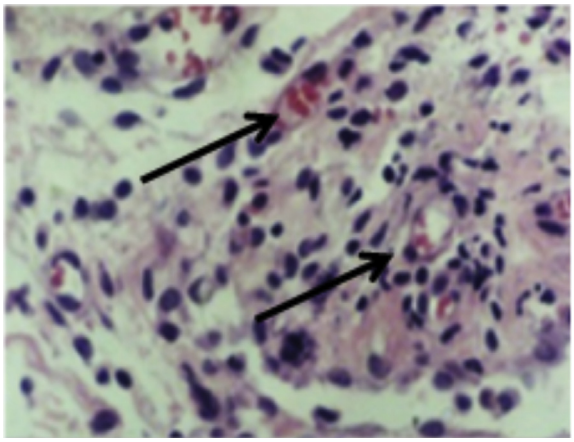

Fig. 2. Photomicrograph of a human leiomyoma xenograft in the peritoneum of a female Wistar rat (Group I) showing neovascularization (arrows) and the absence of cellular atypia after 14 days of immunosuppressant administration [mycophenolate mofetil (40 mg/kg)] and estrogen supplementation (estradiol valerate).

The evaluation of fibrosis using Masson's trichrome staining demonstrated that two rats presented grade III fibrosis and one rat presented grade I fibrosis in group I. In group II, two rats had grade I fibrosis, and one had grade II fibrosis (Table 1).

The H&E analysis revealed the presence of small vessels around the xenografts, which indicates the neovascularization of the transplanted leiomyoma. Cellular atypia and mitosis were not identified in any of the samples (Fig. 2).

When the histological variables necrosis and neovascularization were considered together, a statistically significant difference between the groups was observed, with group I showing evidence of greater implant success compared to group II, $p = 0.039$ (Fig. 3).

Comments

The scientific community has been intensifying its search for improved experimental models, and several new models have been proposed since the *Eker rat* model was first reported. In the Eker model, tumors develop in response to a germline mutation in the tuberous sclerosis 2 (*TSC2*) gene, and this tumor predisposition is inherited in an autosomal dominant pattern [8,23]. However, since this animal model was first reported, newer studies have proposed establishing alternative *in vivo* models.

Hassan et al. described gene therapy in animals implanted subcutaneously with uterine leiomyoma xenografts. In the Hassan study, adenovirus administration was required to maintain the grafted leiomyoma in NOG I mice, a *genetically selected strain* of mice. Tumor vascularization was maintained in these animals by the overexpression of *pro-angiogenic factors* [9].

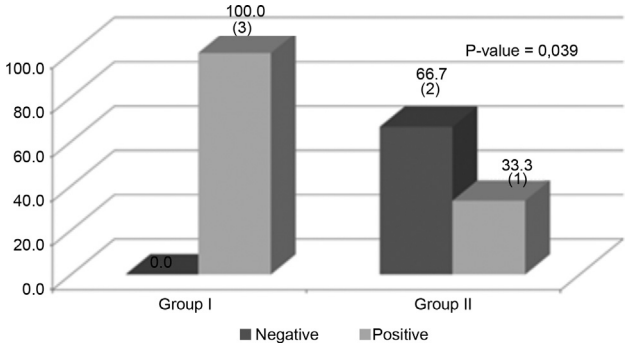

Fig. 3. Association between the examined variables (neovascularization and necrosis) in the human uterine leiomyoma xenograft four weeks after transplantation into the parietal peritoneum (Group I) or subcutaneous tissue (Group II) of female Wistar rats immunosuppressed with mycophenolate mofetil (40 mg/kg) and supplemented with estrogen (estradiol valerate) throughout the entire experiment.

Suo et al. proposed an experimental model of leiomyoma that was used for the first imaging analysis of a leiomyoma implant and the development of uterine fibroids. The authors compared two different fluorescence imaging techniques. The Suo model involved the use of immune-compromised mice, gene therapy of uterine *leiomyomas*, exogenous estrogen supplementation and verification using diagnostic imaging. Although this model, which used gene therapy and a sophisticated technological apparatus, proved to be reproducible, its high cost limits its widespread use [24].

Ishikawa et al. used an abdominal approach to study leiomyoma in rats. Leiomyoma cells were grafted under the renal capsule of immunodeficient NOD/SCID/IL-2R gamma null (NSG) mice. The implant location under the renal capsule is well vascularized, a fact that facilitates the implantation process. Despite satisfactory results, the limiting factors of the model include the risks associated with this technique. In addition, an expensive genetically selected *strain* of mouse is required [25].

Due to the limiting factors of their previous study, Ishikawa et al. subsequently reported using a mouse strain that was less immunodeficient than the NSG, NOD/SCID, BALB/c and NOD/SCID mice [25]. These authors reported advantages such as better graft implants and lower costs. In their second study, mice were ovariectomized and supplemented with intramuscular estrogen. The effectiveness of the implant was evaluated by measuring the activity of the estrogen receptor using immunohistochemistry. The primary limitation of this newer model is the fact that successfully targeting the implantation site requires good surgical skills.

Tsujji et al. transplanted human uterine leiomyoma xenografts subcutaneously in an immunodeficient mouse strain (NOD/SCID/γ c-null: NOG) and reported several practical advantages in the technique itself and in the approach of leiomyomas, which led to lower risk levels associated with the implantation technique for these animals. However, as described above, this model requires technology to produce a *genetically selected strain* of immunodeficient mice, which makes it more difficult to implement and reproduce [10].

In the present study, the histological variables of necrosis and tissue neovascularization demonstrated that human uterine leiomyoma xenografts transplanted into rats via a peritoneal route of administration are superior to those delivered via a subcutaneous route. Wistar rats and the histopathological evaluation techniques used in this experiment are relatively inexpensive and are accessible for our community. Regarding immunosuppression, a non-genetic model was proposed that features a low cost and accessible oral supplementation. Mycophenolate mofetil (MMF) was chosen as the immunosuppressive agent because it has been readily available in Brazil since 1996. In addition, its properties are known and its effects on the induction and maintenance of xenografts have been well documented, thus reducing the risk of acute rejection [17].

In the majority of the studies addressing experimental leiomyomas [8,9] estrogen has been administered either subcutaneously or intramuscularly. Because our group did not have access to injectable estrogen, oral supplementation was used. For this supplementation method, estrogen was diluted in the water that was consumed daily by the rats. Specifically, 1 mg of estradiol valerate was diluted in 250 ml of water once daily and provided for the rats to drink. The daily consumption of water was observed to be approximately 10–20 ml/day, which is consistent with the water-consumption levels reported in the literature. The daily intake of estradiol valerate per rat was estimated to be approximately 0.04–0.08 mg/day.

The current pilot study demonstrates the viability of implanting leiomyomas, that the peritoneal approach is more appropriate and describes a simplified protocol for studying experimental leiomyomas in Wistar rats immunosuppressed with mycophenolate mofetil. This study may contribute to the development of new therapeutic strategies for uterine leiomyoma.

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